A Deficiency in DNA Repair and DNA-PKcs Expression in the Radiosensitive BALB/c Mouse

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Abstract

We have studied the efficiency of DNA double strand break (DSB) rejoining in primary cells from mouse strains that show large differences in in vivo radiosensitivity and tumor susceptibility. Cells from radiosensitive, cancer-prone BALB/c mice showed inefficient end joining of γ-ray-induced DSBs as compared with cells from all of the other commonly used strains and F1 hybrids of C57BL/6 and BALB/c mice. The BALB/c repair phenotype was accompanied by a significantly reduced expression level of DNA-PKcs protein as well as a lowered DNA-PK activity level as compared with the other strains. In conjunction with published reports, these data suggest that natural genetic variation in nonhomologous end joining processes may have a significant impact on the in vivo radiation response of mice.

Introduction

DNA DSBs are the most deleterious form of DNA damage and if left unrepaired or misrepaired can lead to cell death, mutation, and neoplastic transformation (reviewed in Refs. 1 and 2). Mammalian cells repair DNA DSBs by at least two mechanisms, i.e., homologous recombination and NHEJ. The latter mechanism is known to be the primary mode of DSB repair in mammalian cells (2). Although cancer-susceptible BRCA1, BRCA2, and Nijmegen breakage syndrome genes are believed to be associated with recombinational repair of DSBs (3, 4), information is limited on the relationship between NHEJ deficiency, radiosensitivity, and cancer proneness.

Differences in radiosensitivity and tumor susceptibility among various strains of mice are well recognized (5, 6). Among several commonly used inbred mouse strains, BALB/c has been consistently found to be unusually sensitive to the lethal effects of radiation (5, 7, 8) and to the development of various types of spontaneous as well as radiation-induced solid tumors (6). Clonogenic assays of cell survival in jejunal crypt cells showed a greatly increased radiosensitivity in BALB/c mice compared with C57BL/6/BALB/c hybrids (9). It also has been shown that the reduction in the lethal effects of radiation as a result of lowering the dose rate is substantially reduced in BALB/c as compared with C57BL/6 mice (10). This dose rate effect is commonly attributed to the operation of repair processes. Cellular studies on radiation-induced murine mammary cancer have demonstrated that strain-dependent differences in susceptibility result from differences in sensitivity to neoplastic initiation (11). More recently, this laboratory has shown that mammary cells from BALB/c mice are more susceptible to radiation-induced genomic instability than those from C57BL/6/F1 hybrids of these two strains (12, 13). These observations support the hypothesis that in certain genetic settings, such instability is associated with early events in radiation-induced tumorigenesis (12–14).

Cells derived from highly radiosensitive SCID mice have a DNA DSB repair deficiency caused by a mutation in the DNA-PKcs gene (15–17); they also show minisatellite instability (18) and increased susceptibility to neoplastic transformation and genomic instability (19). The defect for numerous independently isolated radiosensitive mutant cell lines has also been traced to involve DNA-PKcs (2). However, depending on the mutation, the phenotypic defect has not always been as severe as that for SCID cells (e.g., irs 20 cells; Refs. 20 and 21). For this reason, we first chose to extend the phenotypic characterizations of these mouse strains showing differences in radiosensitivity to include the status of DNA DSB rejoining and the DNA-PK protein complex in kidney cells freshly isolated from BALB/c, C57BL/6, CXB6F1 (F1 hybrid of BALB/c and C57BL/6), AJJ, C3H, and DBA strains. Our findings demonstrate that BALB/c carries a defect in DNA DSB rejoining that is quantitatively different from that of SCID mice. Although these observations do not, of course, establish a cause and effect relationship, the possible implications of these data for the in vivo radiation response of mice are discussed in the context of postirradiation lethality, apoptosis, and tumorigenesis.

Materials and Methods

Animals. BALB/c ByJ, C57BL/6 ByJ, CB6F1 (BALB/c female × C57BL/6 male; referred to as F1 in the report), SCID (in BALB/c and C57BL/6 genetic backgrounds), AJJ, C3H/HetJ, and DBA2J were obtained from Jackson laboratory and maintained in the University of Texas Medical Branch animal resource center.

Cell dissociation. Female mice, 10–26 weeks of age, were anesthetized, and the kidneys were extracted. Cell suspensions were prepared by mincing the kidneys and incubating fragments in 199 medium containing collagenase (Type III; 200 units/ml) at 37°C for 4–5 h with gentle agitation. The resultant cells were extensively washed in α-MEM medium containing triton X-100, 0.1% to wash out the residual collagenase. The cells were then suspended with 0.1% trypsin, and the kidneys were incubated in 0.1% trypsin for 5–10 min before each experiment.

Irradiation. Irradiation was carried out using a 137Cs irradiator with a dose rate of 6.70 Gy/min at 0°C. For a study of DNA DSB rejoining kinetics over periods >2 h, cells were irradiated at room temperature.

DNA DSB assay. DNA DSBs were measured by clamped homogeneous electric field gel electrophoresis (CHEF). Cells were incubated with 0.01 μCi/ml [3H]thymidine and 5 μM cold thymidine for at least 2 days before each experiment. Cells were irradiated in plastic dishes, and DNA repair was allowed to proceed at 37°C in air:CO2 (95:5). At each repair point, cells were

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3 The abbreviations used are: DSB, double strand break; NHEJ, nonhomologous end joining; SCID, severe combined immunodeficient; FAR, fraction of activity released.

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trypsinized and washed, and the resultant cell pellet was immediately embedded in 0.5% agarose (Incert agarose; FMC). These agarose samples were cut and immersed in an ice-cold lysis solution containing 0.5 mM EDTA, 0.01 mM Tris, 2% Sarcosyl, and 0.2 mg/ml proteinase K (pH 8.0) for 1 h, followed by an overnight incubation at 50°C. For the initial (0-h) repair point, cells were embedded in agarose and irradiated in ice-cold medium, followed by an immediate lysis at 0°C for 1 h and 50°C overnight. After the overnight lysis, samples were washed for 1 h in 0.1 mM EDTA, 0.01 mM Tris at pH 8.0 and treated with 0.1 mg/ml RNase A for 1 h at 37°C.

Electrophoresis was carried out in 0.5 X TBE buffer (45 mM Tris, 45 mM boric acid, and 1.5 mM EDTA, pH 8.2) in a clamped homogenous electric field gel box (Bio-Rad) at 0.8% agarose gel (Bio-Rad) at 14°C. The applied voltage was 200 V with 60 s pulse time for the first 9 h, followed by a 120 s pulse time for the last 15 h. After electrophoresis, gels were stained and cut to separate the plug from the lane for each sample. The 14C activity of each piece was subjected to liquid scintillation counting. The enzymatic activity of DNA-PK was washed, air-dried according to the manufacturer’s instructions, and then sub-

Western Blotting. For analysis of Ku protein, cells were lysed in buffer containing 25 mM Tris (pH 7.4), 50 mM NaCl, 0.5% sodium deoxycholate, 2% NP40, 0.2% SDS, 1 mM phenylmethylsulfonyl fluoride with 50 µg/ml apro-

Protein Lysate: Western Blotting. For analysis of Ku protein, cells were lysed in buffer containing 25 mM Tris (pH 8.0), 125 mM NaCl, 0.025% Tween 20 and incubated in blocking buffer containing 25 mM Tris (pH 8.0), 125 mM NaCl, 0.025% Tween 20, and 5% dry milk for 1 h at room temperature. The primary antibodies used were Ku 70 and Ku 80 polyclonal goat antimouse antibodies (Santa Cruz Biotechnology) for Ku (1:200 dilution) and mouse monoclonal DNA-PK Ab-4 for DNA-PKcs (1:500 dilution; Lab Vision, Fremont, CA) and applied for 1 h at room temperature. The membranes were washed and incubated with secondary antibodies labeled with horseradish peroxidase, diluted (1:2000) in blocking solution for 1 h. The secondary antibodies used were donkey anti-antibody IgG for Ku (Santa Cruz Biotechnology) and antimouse F(ab’2) for DNA-PKcs (Amersham). Subsequently, the membranes were washed and incubated in ECL Western blotting detection reagent (Amersham RPN 2106) for 1 min. The blot was exposed to X-ray film for 5 min. All of the membranes used for the blot were stained with Ponceau S solution (Sigma) to ascertain that an equal amount of protein was loaded to each lane.

In Vitro Protein Kinase Assay. DNA-PK kinase activity was measured using a “pull-down” assay (24) with modification. Briefly, whole-cell extracts were prepared from primary mouse cells as described (25). One hundred µg of protein lysate were mixed with 40 µl of 1:1 slurry of DNA-cellulose and incubated on ice for 15 min, followed by a brief centrifugation. The supernatant was re-extracted with 20 µl of DNA-cellulose. The pellets containing DNA-cellulose bound DNA-PKcs were combined, washed, and resuspended in distilled water. The samples were then incubated with biotinylated peptide substrate supplied in the Sigmatek DNA-PK kinase assay kit (Promega) in the presence of [γ-32P]ATP for 5 min at 30°C. A fraction of reactant was spotted onto the SAM® membrane (supplied in the kit). The membrane was washed, air-dried according to the manufacturer’s instructions, and then subjected to liquid scintillation counting. The enzymatic activity of DNA-PK was calculated and expressed as in pmol ATM min^{-1}mg^{-1} of protein.

Results

The kinetics of DNA DSB end-joining in irradiated primary cells from various mouse strains are shown in Fig. 1. The percentages of remaining DSB damage (normalized FAR values) are shown as a function of postirradiation times. In these experiments, cells were incubated at 37°C after exposure to γ radiation (50 Gy), and at each time point, samples were prepared and lysed immediately. A significant difference in repair kinetics was observed in cells from SCID and BALB/c strains. Cells from C57BL/6, A/J, C3H, DBA, and F1 cells rejoined DNA DSBs very efficiently. As expected, cells from SCID mice showed severe deficiency of DSB rejoining (26), whereas BALB/c cells gave an intermediate response.

It has been suggested that the DNA-PK complex, which consists of a heterodimer Ku (Ku 70 and Ku 80) and M_{r} 460,000 DNA-PKcs, plays a significant role at the initial stage of DNA DSB end joining as well as in the V(D)J recombination process (1, 2, 27). If one of these components is functionally defective in cells (e.g., from SCID mice), they can manifest as a defect in NHEJ, leading to severe ionizing radiation sensitivity and/or V(D)J recombination, resulting in immune deficiency.

Data on Ku 70, Ku 80, and DNA-PKcs protein expression obtained by Western blotting in extracts from BALB/c and C57BL/6 mice are shown in Fig. 2A. The expression levels of Ku proteins are similar in all of the nonirradiated and irradiated BALB/c and C57BL/6 samples, and the expression does not seem to be affected significantly by radiation. By contrast, there is a large difference in the expression level of DNA-PKcs between BALB/c and C57BL/6 cells. Greatly reduced expression of the original size DNA-PKcs (M_{r} 460,000) as well as an excess of degraded cross-reacting protein (a band seen around M_{r} 202,000) has been observed in all of the BALB/c samples. Radiation does not seem to have a measurable effect on the expression of this cross-reacting material 1 h after irradiation. Cleavage of DNA-PKcs molecules into M_{r} ~240,000 fragments has been reported with exposure to various cytotoxic agents in Burkitt lymphoma cells (23), and the possibility of a novel protein of this size has also been suggested (16). Fig. 2B is a Western blot for DNA-PKcs using cell extracts from all of the strains used in the DNA DSB repair experiment (Fig. 1). As expected, no DNA-PKcs expression was observed in SCID, and BALB/c showed a weak expression, whereas all of the
other strains showed similar substantial DNA-PKcs levels with F1 being slightly lower than C57BL/6 and the other four strains. This result is consistent with that of DNA DSB rejoining kinetics shown in Fig. 1.

To further characterize DNA-PK protein in these mouse strains, kinase activity levels were measured in whole-cell extracts obtained from these strains in vitro. For these functional assays, the sensitivity was increased using the method of Finnie et al. (24). Fig. 3 summarizes the results of the DNA-PK activity assay for all of the mouse strains and indicates a substantially lower activity level for BALB/c and the lowest level for SCID mice, as compared with all of the other strains. Although the contribution of kinase activity level to DNA DSB repair is not yet established, the functional assay data of Fig. 3 are consistent with those on protein expression (Fig. 2) and DNA DSB rejoining results (Fig. 1).

Discussion

The results presented here provide the first evidence that BALB/c mice have a defect in DNA DSB rejoining; specifically, that involving NHEJ. Although the repair pathway is expected to be different, one report suggests similar strain differences in the repair of benzo[a]pyrene-DNA adducts (28). It has also been suggested that cells from the BALB/cAnPt strain rejoin DNA DSBs more slowly than other strains based on studies of chromosome breaks in irradiated cells during the G2 stage (29).

The degree of severity of the DNA DSB repair defect in highly radiosensitive SCID mice is substantially greater than that of BALB/c mice (Fig. 1), implying that BALB/c radiosensitivity is likely to be less pronounced. Importantly, however, the in vivo radiosensitivity of BALB/c may be distinguished from that of other inbred mouse strains. The interstrain whole body X-irradiation studies of Grahn and Hamilton (7), Sandler and Gowen (8), and Roederick (5) spanning almost 30 strains consistently showed BALB/c to be maximally susceptible to the lethal effects of radiation, largely a consequence of cell killing in critical organs. In these studies, C57BL/6 mice fell into the radioresistant category (e.g., Fig. 1 in Ref. 7). Further, Kallman (10) showed that the loss of effectiveness commonly observed with a reduction in dose rate and generally attributed to repair processes is greatly reduced for the lethal effect of radiation in BALB/c as compared with C57BL/6 mice. Regarding radiosensitivity at the cellular level, Hanson et al. (9) showed a large difference in the dose-survival response of jejunal crypt cells by clonogenic survival measurements for BALB/c versus C57BL/6/BALB/c hybrid mice. The former were more radiosensitive by nearly a factor of two (9).

Almost all in vivo and cellular measures of radiosensitivity will be quantitative genetic traits influenced by the specific distribution of variant germ-line sensitivity/resistance alleles between different mouse strains. On this basis, the in vivo data on postirradiation lethal effects might be explained if BALB/c were to carry a major sensitivity allele with respect to cellular radiation response that distinguishes it from most other commonly studied mice. Some support for this contention is available from the genetic investigations of Mori et al. (30) on mouse loci that determine the in vivo apoptotic response of thymocytes to X-rays. In these studies, BALB/c was shown to carry a recessive chromosome 16-encoded variant gene (Rapop1) that represents a major determinant of susceptibility to apoptotic response. The fact that Rapop1 was mapped by Mori et al. (30) to the same mouse chromosome 16 genomic segment now assigned to the DNA-PKcs (Prkdc) gene provides further circumstantial evidence to suggest a direct genetic association between the recessive DNA DSB repair/DNA-PK protein expression phenotype of BALB/c and in vivo radiosensitivity.

The extent of the data available on interstrain variation in radiation tumorigenesis in mice does not allow specific genetic correlations to

Fig. 2. A, expression of Ku 70, Ku 80 and DNA-PKcs protein as detected by Western blot in whole-cell extracts derived from BALB/c and C57BL/6 primary cells. The first samples (designated 0) were prepared without radiation, and the second and the third samples (designated 5 and 50) were prepared with 5 and 50 Gy γ-irradiation. B, comparison of DNA-PKcs protein expression as detected by Western blot in extracts derived from A/J, BALB/c, SCID, C3H, C57BL/6, DBA, and F1 (F-1) primary cells.
be suggested, but nevertheless, the quantitative tumorigenesis data of Storer et al. (6) on C57BL/6, RFM, C3H, and BALB/c mice are not inconsistent with the view that a tumor susceptibility allele is carried by BALB/c.

Relating DNA DSB repair deficiency and DNA-PKcs expression in mice to patterns of tumorigenesis is not straightforward. SCID mice are characterized by excess lymphomas rather than solid cancers (31), as are DSB repair-deficient Ku 70/−/− knockout (32). Whether this lymphoma susceptibility reflects the severity of the repair defect and poor cell survival or, alternatively, the influence of genetic background remains to be resolved. In the case of BALB/c, which has an unremarkable lymphoma incidence (6), the partial defect in DSB repair may, in principle, target different organs.

The data presented here provide initial evidence that a partial BALB/c defect in DNA-PKcs protein expression may underlie the DNA DSB repair phenotype. Instability of DNA-PKcs suggested by the data of Fig. 2, leading to reduced kinase activity (Fig. 3), would be expected to lead to a reduction in the efficiency of DNA DSB repair, particularly in cells where DNA-PKcs tended to be expressed at low levels (33). However, whether these cellular phenotypes and their associations with in vivo radiation response are a reflection of specific variation at the mouse Pkd locus will demand genetic mapping of controlling loci and sequence analysis of DNA-PKcs in appropriate strains.

In conclusion, the studies reported here show that natural genetic variation between inbred mice can provide the means to explore the relationships between the expression of specific proteins, DNA repair deficiency, genomic instability, and in vivo radiation response.

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References

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